IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Haruo SUGIYAMA

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For: DIMERIZED PEPTIDE

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents,
Washington, D.C.

Sir:

The undersigned, Haruo Sugiyama, M.D., Ph.D., residing at 2-19-30, Senbanishi, Minoo-shi, Osaka, Japan, do hereby declare and state as follows:

1. My Curriculum Vitae including a list of my publications published in English, are attached hereto as Appendix.

2. On the basis of the qualifications set forth in my Curriculum Vitae, I submit that I am an expert in the fields of cancer biology and immunology. I have long been engaged in research on tumor antigens and identified several novel antigen peptides derived from WT1 protein that is an expression product of tumor suppressor gene WT1 of human Wilms' tumor, and conducted clinical studies.

3. I have studied the Office Action issued on July 18, 2007 and understood what is the essence of the Office Action.

4. I am one of inventors of the US Patent Application number

10/541821 (hereinafter, "present application") and am familiar with the scientific and practical significance of the invention.

5. I have performed, or supervised the performance of, the experiments described in the following paragraphs in support of patentability of the above-identified patent application.

6. Experiments

6.1 Experiment 1: Cross-reactivity of CTLs Induced by a Peptide Homodimer of the Present Invention

Method

According to the method described in the present application, emulsion of a peptide dimer of EXAMPLE 1 was prepared and administered to HLA-A24 transgenic mice in a similar manner to TEST EXAMPLE 1. Two mice were used. Seven days after the administration, the spleen was removed and splenocytes were prepared. Number of CTLs specifically react with a peptide and generate interferon-γ was counted by ELISPOT method. Splenocytes were seeded into a 96-well ELISPOT plate at 5×10^5 cells/well and thereto was added a peptide monomer (SEQ ID NO: 44), a natural-type peptide (SEQ ID NO: 11) having a sequence wherein the second amino acid from the N-terminus is methionine, or an influenza virus (Flu)-derived peptide (ASNENMETM) having a sequence irrelevant to WT1 peptide. After culturing overnight, cells were removed by washing. Interferon-γ was detected by coloring, and spots were counted to obtain the number of CTLs. The results are shown in Figure 1.

Results

As can be seen from Figure 1, administration of the peptide dimer of the present invention induced CTLs which recognize the peptide monomer or the natural-type peptide monomer. Since cancer cells expressing WT1 present natural-type peptides, the present experimental results demonstrate that the peptide dimer of the present invention is useful as a pharmaceutical composition for inducing CTLs which kill cancer cells.

Figure 1

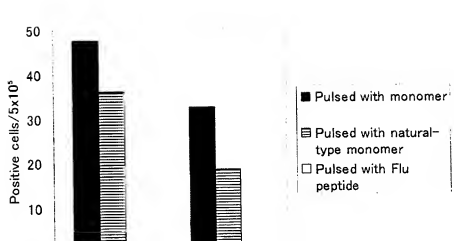


Figure 1: The reactivity of CTLs induced by a peptide dimer (dimerized peptides of SEQ ID NO: 44) in transgenic mice to respective peptides.

6.2 Experiment 2: Stability in Blood

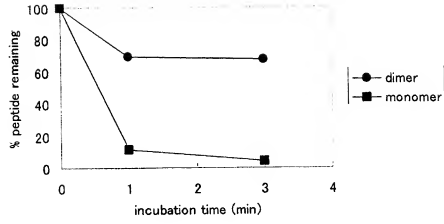
Method

Monomers and homodimers of the peptide (SEQ ID NO: 44) were dissolved in DMSO separately to obtain a 2 mM peptide solution. The monomer solution was diluted by mouse serum to prepare a 100 μ M monomer peptide solution. The dimer solution was diluted by mouse serum to prepare a 50 μ M dimer peptide solution. The amount (percentage) of peptides remaining unchanged was measured by reversed phase high performance liquid chromatography at the time of addition of mouse serum (0) and 1 and 3 minutes thereafter. The results are shown in Figure 2.

Results

As can be seen from Figure 2, at least 65 % of homodimers remained unchanged after 3 minutes; however, not less than 5 % of monomers remained unchanged. These results show that homodimers are far more stable than monomers in blood.

Figure 2



6.3 As shown in Experiments 1 and 2, the homodimer of the present invention can induce CTLs having cross-reactivity and being capable of recognizing monomer peptides of not only the variant type (SEQ ID NO: 44) but also the natural-type (SEQ ID NO: 11), and is far more stable than monomer in blood.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: January 15, 2008

Haruo Sugiyama

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Educations / Training

1969-1975	Student, Osaka University Medical School , M.D.
1975-1979	Ph. D. course of Institute for Microbial Diseases , Ph.D
1979-1980	Research Fellow, Institute for Microbial Diseases
1980-1983	Medical Fellow, Osaka University Hospital
1983-1994	Assistant Professor, Department of Medicine III, Osaka University Medical School
1994-1995	Lecturer, Department of Medicine III, Osaka University Medical School
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APPENDIX

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Teaching the Immune System To Fight Cancer

Certain molecules on tumors can serve as targets for attack by cells of the immune system. These tumor-rejection antigens may provide a basis for precisely targeted anticancer therapy

by Thierry Boon

At its best, the immune system is the ideal weapon against infectious disease. It eliminates viruses and bacteria that invade the body and kills infected cells, yet it leaves healthy tissue intact. The system is so precise because it responds only to specific targets called antigens: molecules or fragments of molecules that belong to the foreign invaders. In general, antibody molecules inactivate pathogens and toxins that circulate in body fluids, whereas white blood cells called cytolytic *T* lymphocytes destroy ("lyse") cells that have been penetrated by viruses.

The specificity and power of the immune system have not escaped notice of cancer researchers. Assuming that *T* lymphocytes might be able to eradicate cancer cells as effectively as they lyse virus-infected cells, investigators have long hoped to identify tumor-rejection antigens: structures that *T* lymphocytes can recognize on tumor cells in the body. These workers reasoned that antigens appearing exclusively (or almost exclusively) on cancer cells could be manipulated in ways that would trigger or amplify a patient's insufficient immune reaction to those targets.

Definitive evidence that tumor-rejection antigens exist on human tumors has been elusive. Yet in the past few years, my colleagues and I at the Ludwig Institute for Cancer Research in Brussels have gathered unequivocal proof that many, perhaps most, tumors do indeed display such antigens. Equally important, we have developed ways to isolate genes that specify the structure of these antigens. Moreover, we and others have seen indications that *T* lymphocytes that normally ignore existing tumor-rejection antigens can be prodded to respond to them. Hence, the design of therapies to generate such *T* cell responses to well-defined tumor-rejection antigens has finally become feasible.

The first clues that tumor-rejection antigens sometimes arise on tumors were uncovered in the 1950s, before the distinct roles of antibodies and *T* cells were elucidated. Several researchers—notably E. J. Foley of the Schering Corporation in Bloomfield, N.J., Richmond T. Prehn and Joan M. Main of the National Cancer Institute and George Klein of the Karolinska Institute Medical School in Stockholm—had generated cancers in mice by treating the animals with large doses of a carcinogenic compound. When the mice were freed of their tumors by surgery and subsequently injected with cells of the same tumor, they did not suffer a recurrence. The mice did acquire cancer after being injected with cells from other tumors, however. Those observations suggested that cells of carcinogen-induced tumors carry antigens that can elicit a response by the immune system.

For about 20 years after those pioneering experiments were completed, hope ran high that human cancers, too, might bear tumor-rejection antigens. The prospect for antigen-based therapy seemed even better when, toward the end of that period, *T* lymphocytes were found to be particularly important for ridding the body of abnormal cells. Jean-Charles Cerottini and K. Brunner of the Swiss Institute for Experimental Cancer Research in Lausanne showed that when mice rejected tissue transplanted from an unrelated donor, the animals produce cytolytic *T* lymphocytes that can destroy cells from the transplant. By then it was apparent as well that when the specialized antigen receptors on cytolytic *T* lymphocytes bind to foreign antigens on a cell, the lymphocytes both lyse the cell and multiply, amplifying the immune reaction. These discoveries intimated that cancer researchers might make major strides if they concentrated on finding the antigenic targets of cytolytic *T* lymphocytes and on augmenting the activity of the cytolytic cells.

In the mid-1970s, however, experiments reported by Harold R. Bennett, then at Mount Vernon Hospital in London, ushered in an era of pessimism. In contrast to the earlier experiments, which examined tumors induced by exposure to massive doses of carcinogens, Hewitt looked for evidence of tumor rejection antigens on spontaneously arising malignancies. His careful work, conducted on many types of cancer, strongly suggested that spontaneous tumors in mammals did not evoke any immune rejection. Hence, he argued, the observations made in the earlier studies had little relevance to human tumors: people are rarely exposed to the high levels of carcinogens with which scientists produce malignancies in the laboratory.

Reasonably, many investigators then turned their attention elsewhere. Yet between 1972 and 1976 my colleagues and I had seen indications that tumor-rejection antigens were present on several mouse tumors that failed to elicit

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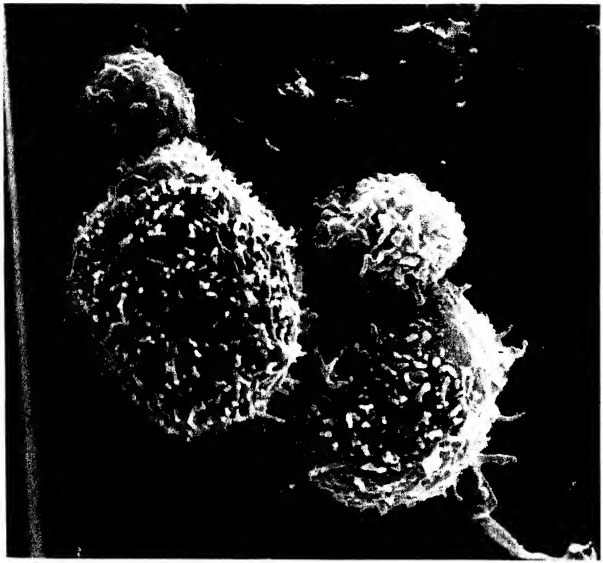


WHITE BLOOD CELLS called cytolytic *T* lymphocytes (*small spheres*) are attacking two cells from a mouse tumor called P815 (*large spheres*). Such lymphocytes bind to tumor cells when they recognize specific targets known as tumor-rejection antigens on the cell surface. Investigators have now found ways to identify the antigens with certainty. They hope to incorporate such antigens into therapies that will incite a patient's own *T* lymphocytes to eradicate tumors.

an immune rejection response. In addition, we discovered that the initially ineffective antigens could become useful targets for a defensive assault if the immune system were somehow made more aware of their existence. And so, even after Hewitt published his data, we remained hopeful that immunotherapies based on tumor-rejection antigens might be possible for humans.

As often happens in science, we were studying a totally unrelated problem in 1972 when we stumbled onto those first clues. We were trying to identify genes that control the way cells in mammalian embryos differentiate to become the specialized cells of mature organisms. My colleague Oddie Kellermann and I, then at the Pasteur Institute in Paris, had exposed a culture of mouse tumor cells to a potent mutagen, a compound that introduces random, permanent changes (mutations) in genes. Then we put individual treated cells in separate plastic dishes and allowed them to proliferate so that each dish eventually contained a population of identical cells (a clone). That done, we transferred the clonal populations into mice and examined the cell types present in the tumors that resulted.

To our disappointment, the experiments did not lead to a better understanding of the mechanisms of differentiation. But they did turn up a highly intriguing phenomenon. The original, or parent, tumor cells (those not yet exposed to the mutagen) almost always yielded cancerous growths when injected into mice. Yet many of the mutagen-treated clones produced no malignancies. Although I was a geneticist by training and knew little about cancer, I felt impelled to find out why the mutagen-treated cells did not form tumors. For simplicity's sake, my associates and I referred to cell clones that failed to generate tumors as tum$^-$ variants.

We found that the tum$^-$ variants

caused by cancer because the immune system of the injected mice had destroyed them, much as it might reject a mismatched kidney transplant. We found as well that the rejection occurred because the mutagen induced the tum- cells to display one or more antigens (tum- antigens) that elicited a potent *T* lymphocyte response; these tum antigens were not present on the parental tumor-inducing (tumorigenic) cell line and appeared to be different for every tum- variant.

The results were interesting by themselves, but what truly captivated us was a second finding I obtained with Aline VanPel after we joined the International Institute of Cellular and Molecular Pathology (ICP) in Brussels. As was true of the spontaneous cancers studied by Hewitt, the cells of our original tumor were totally incapable of eliciting an immune attack. Yet often when we injected these cells into mice that had rejected one or another tum- variant, no cancer developed. In mounting an immune response to a tum- variant, the mice somehow acquired resistance to the original tumor cell. The mice did not resist unrelated cancers, however, indicating that rejection of the original tumor cells was caused by an antigen shared by the tum- variant and its parent but not by other cancers.

Our findings were later confirmed in several follow-up studies involving many different mouse tumors. Most important, Van Pel observed that she could reproduce our results with the very spontaneous tumors Hewitt had examined. Clearly, the conclusion that spontaneous cancers did not display tumor-rejection antigens had to be revised.

No one has fully explained how tum- variants manage to induce a powerful immune response to the initially inert (or mild or weak) antigens on the original cells. We suspect that small proteins called interleukins play a role. A lymphocyte that has bound to an antigen releases interleukins. These proteins, in turn, promote proliferation of that lymphocyte and nearby ones such as those bound to another antigen on the same tumor cell or on neighboring cells. It seems probable that the tum- antigens are potent enough to spur *T* lymphocytes to kill tum- cells and to multiply rapidly even in the absence of processing interleukins in the local environment. These lymphocytes then produce interleukins, which help other *T* cells become activated by weak tumor-rejection antigens. Consistent with this view is the fact that in recent years several research groups have modified tumor cells to secrete interleukins. In many instances the workers have seen a considerable increase in the immune response to the tumors.

By the early 1980s, then, our collected evidence suggested the following conclusion: mouse tumors that normally fail to elicit a buildup of *T* lymphocytes nonetheless often carry weak antigens that can become targets for an effective immune assault. Because the immune system of mice is much like that of humans, the data implied that human tumors might be antigenic as well. If so, they might be susceptible to immunotherapy that artificially induced an antigen-specific attack. In other words, immunotherapy for humans was a reasonable goal. At that point, we decided to apply all the forces of our laboratory to the study of tumor-rejection antigens.

Before considering therapy, we would have to identify specific tumor-rejection antigens. All earlier attempts to isolate such structures directly from cell membranes in human and mouse tumors had failed. We therefore decided to try an alternative approach: cloning, or isolating, the genes that direct construction of the antigens. Unfortunately, no one had yet come up with a good way to perform the task. And so in 1983 my colleagues and I, by then members of the Ludwig Institute, set out to develop a method of our own. It took us four years to devise an approach that would work in a test system [*see box on opposite page*].

In our first successful cloning effort, we isolated the gene for the tum- antigen appearing on the cells of a mouse tum- variant. Of course, tum- antigens are not true tumor-rejection antigens, because they are artificially induced to appear on cultured tumor cells and are not found on cancers in the body. But, as will be seen, they were useful for our trial run. We generated the tum- variant from a cell line that was derived from a mastocytoma (mast cell tumor) named P815. The original P815 cell line was appealing for our purposes because the cells replicate rapidly and indefinitely in the test tube. In addition, tum- variants of P815 cells provoke cytolytic *T* lymphocytes into a strong, readily detectable response.

Our gene-cloning plan relied first of all on having a good supply of cytolytic *T* cells reactive to the tum- antigen of the variant. The *T* cells would later lead us to the gene for the antigen. To acquire the cytolytic cells we injected the P815 tum- variant into mice. Then we removed the spleen (a repository of lymphocytes) from animals that rejected the variant. We knew that if the lymphocytes from these immunized animals were exposed to killed cells of the variant, cytolytic *T* lymphocytes specific for the variant would multiply. Accordingly, other lymphocytes would disappear. (Tumor cells would be killed to prevent them from overtaking the culture.) When we had a supply of cytolytic *T* lymphocytes, of which some responded to the tum- antigen and others to tumor-rejection antigens present on all P815 cells, by placing individual lymphocytes in laboratory dishes and allowing them to replicate separately, we obtained several clones that would live only for tum- variant and could be made to multiply indefinitely in laboratory dishes. We chose one of the clones directed against the tum- antigen to use in the quest for the gene.

In outline, the plan for isolating the gene for the tum- antigen was straightforward. We intended to collect all the genetic material of the variant. Next we would link fragments of this DNA to pieces of bacterial DNA, which as will later serve as labels to help retrieve the gene of interest. We would introduce the fragments into cells that do not normally produce the tum- antigen. Then we would test the ability of each of these cells to stimulate our *T* lymphocyte clone. We would know that a recipient cell displayed the antigen and thus had taken up the corresponding gene if the cell spurred the lymphocyte to proliferate. By searching for the bacterial label we had attached to the DNA of the tum- variant, we would locate and retrieve from the DNA of the recipient cell the gene for the tum- antigen.

Although the plan was relatively simple conceptually, the implementation it was quite laborious. Mammalian cells contain approximately 100,000 distinct genes, spread throughout roughly three billion nucleotides (the building blocks of DNA) in the chromosomes inherited from each parent. Because of inadequacies in the techniques available for inserting DNA into recipient cells, we had to create a gene "library" containing millions of copies of each gene. The sections were obtained by sparing fragments of the DNA from the tum- variant and 300,000 plasmids, or circular bits of bacterial DNA; each such plasmid contained about 40,000 nucleotides of inserted tum- DNA (containing an average of one or two genes). After allowing the plasmids to multiply in bacteria, we recovered the DNA.

Next we selected as the recipient a cell type that could incorporate such plasmids into its chromosomes. The original P815 line proved suitable. To ensure that at least one copy of each gene in the tum- variant would be into the DNA of the recipient P815 cells, we

had to mix the recovered plasmids with more than 300 million PS15 cells. We needed that many because we knew only about one in 10,000 of the cells would take up DNA. We also knew that these few cells would accept a lot of DNA: 500,000 nucleotides on average.

Fortunately, we were able to avoid having to test every last cell for its ability to activate the selected clone of *T* lymphocytes. We did so by including in the bacterial DNA a gene that conferred resistance to a particular toxic drug. When we treated the full set of cells with the drug, we eliminated all those that had failed to integrate a plasmid into their DNA. We were thus left with just 30,000 of the original 300 million PS15 cells. By testing small groups of the 30,000 cells, we found the few that stimulated the *T* lymphocytes to multiply. We then homed in on the bacterial DNA of one of these cells and thus picked out the tum- DNA. By repeating much the same process with this DNA fragment, we were soon able to isolate the gene giving rise to the tum- antigen.

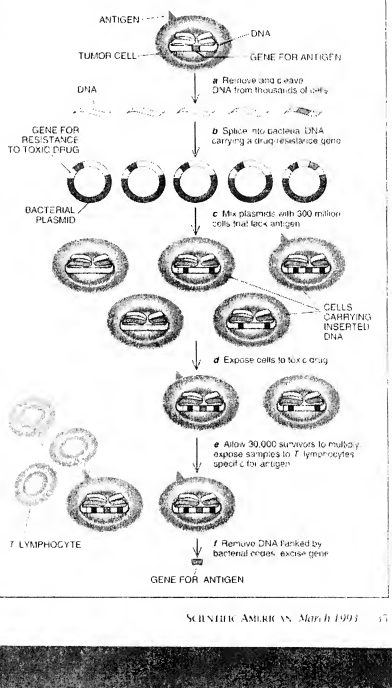
We quickly deciphered the sequence of nucleotides in the gene. The sequence did not resemble that of any gene known at the time. We did find, however, that the gene was expressed not only in the tum- variant but also in the original PS15 cells and in normal mouse tissue. That is, the gene, which specifies the sequence of amino acids to be strung together into a protein, was being transcribed into molecules of messenger RNA that were, in turn, being translated into protein.

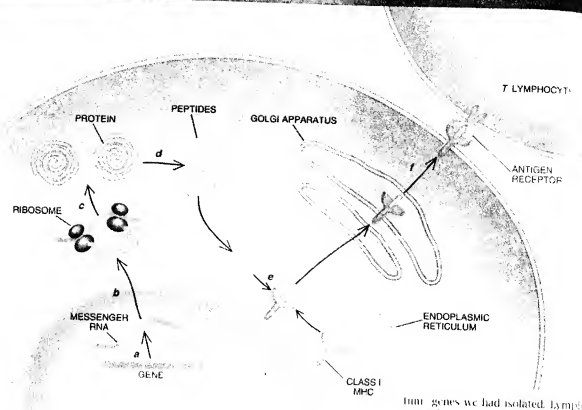
Expression in normal cells meant that our gene specified a standard component of cells. But all was not normal in the tum- variant. There the gene had suffered a point mutation, causing one amino acid to be substituted for another in the protein product. The same was true of two other tum- genes we cloned later. We were puzzled. How could a single amino acid substitution transform a cell's constituent of normal cells into a foreign antigen recognized by cytolytic T lymphocytes?

Just when we were asking this question Alan R. M. Townsend of John Radcliffe Hospital in Oxford, England, and his colleagues made a discovery that led to the answer. In 1986 they demonstrated that cytolytic *T* lymphocytes can often detect viral proteins hidden within cells. In contrast, antibodies respond only to proteins that exert their influence on the cell surface. The *T* cells can accomplish this feat because, in the course of mammalian evolution,

How Genes for Antigens Recognized by *T* Lymphocytes Are Cloned

Cloning, or isolation, of a gene (*red band in nucleus*) for an antigen (*red triangle*) on a tumor cell begins with removal and cleavage of DNA from multiple copies of the cell (*a*). Workers insert the resulting DNA fragments into plasmids (rings of bacterial DNA) bearing a gene (*yellow*) that confers resistance to a toxic drug (*b*). They mix the plasmids with cells that lack the antigen, causing some of those cells to take up one or more plasmids (*c*). Next investigators expose the cells to the toxic drug (*d*), thereby eliminating any cells that have failed to incorporate the plasmid DNA into their own DNA. The surviving cells are allowed to multiply, and samples are exposed to *T* lymphocytes that specifically recognize the antigen of interest (*e*). Any cell that induces a lymphocyte response (such as proliferation) can be assumed to produce the antigen, which means it also harbors the corresponding gene. Hence, researchers remove the foreign DNA from an identical cell, excise the bacterial DNA and fish out the desired antigen specifying gene (*f*).





CELLS PRODUCE ANTIGENS (*red and green complex at top right*) in a multistep process. Once a gene (*red band at bottom*) directs synthesis of a protein (*a–c*), cellular enzymes chop these proteins (*large red coils*) into fragments (*small red bars*) called peptides (*d*). Some of these peptides are then transported into an intracellular compartment (the endoplasmic reticulum) (*e*), where they may combine with so-called class I major histocompatibility (MHC) molecules (*green*). Such peptide-MHC complexes are transported to the cell surface (*f*), where *T* lymphocytes (*orange body at top right*) can examine them.

an elaborate protein surveillance system has arisen. Cellular enzymes routinely chop a fraction of all the proteins in the cytoplasm into small fragments known as peptides. These peptides are transported to a special intracellular compartment, the endoplasmic reticulum. There some of them fit themselves into a groove within specialized proteins known as class I major histocompatibility (MHC) molecules. (In humans, MHC molecules are also called human leukocyte antigens, or HLA molecules.) The MHC peptide complexes move to the surface and become anchored to the cell membrane, ready to be scrutinized by cytolytic *T* cells. Lymphocytes whose antigen receptors can bind to such a complex may then attack the cell. Thus, peptides derived from normal proteins are continuously displayed. This presentation does no harm because of natural tolerance early in life: the body eliminates all *T* lymphocytes that recognize the constituents of the self. But if a peptide is derived from a foreign protein, such as that of a virus hiding within a cell, then a *T* lymphocyte will notice it and attempt to kill the cell.

On the basis of these observations, we surmised that the point mutations in the three tum genes had converted peptides that were not seen by *T* lymphocytes to ones that were seen. To test this idea, we made use of a crucial observation of Townsend and his colleagues. They had found that healthy cells could be rendered instantly recognizable to antiviral cytolytic *T* lymphocytes if the cells were put in a medium containing a synthetic version of a small peptide belonging to a viral protein. Presumably, the healthy cells stimulated the lymphocytes because a few MHC molecules on the surface had taken up the peptides and presented them to the *T* cells.

We conducted similar experiments to reveal the role of tum mutations. We mixed P815 cells with small peptides (of nine to 10 amino acids) coded for by the mutated regions of the three tum genes we had isolated. Lymphocytes that react to tum antigens but normally do not attack P815 cells destroyed the cells. But the lymphocytes did not lyse P815 cells that were mixed with peptides encoded by the normal sequences of the genes. Later we showed that the point mutations in two of the tum genes had rendered the affected peptides capable of binding to MHC molecules. The normal versions of these peptides do not bind and consequently are never displayed to the immune system. For the third mutated gene, the situation was different. The normal version of the altered peptide does in fact bind to MHC molecules. But because it is a constituent of the self, the process of natural tolerance had eliminated any *T* lymphocytes responsive to it. The mutation changed the shape of the exposed part of the peptide so that the peptide could now be detected by an existing *T* cell population.

Conversely, a mutation in virtually any gene can result in the appearance of a new antigen on a cell. According, an infinite variety of antigens can be produced by random mutations. The diverse antigens that appear on rodent tumors induced by chemical carcinogens probably arise through such a mechanism. In addition, mutations can occasionally transform normal genes into ones that cause cancer (oncogenes). Some of these oncogenic changes may well generate antigenic peptides that will one day serve as targets for specific immunotherapy.

Having demonstrated the merit of our

cloning technique, we set about isolating a gene of a bona fide tumor rejection antigen—one present on a cancer that grows in an animal. Fortunately, we had at our disposal a cytolytic *T* lymphocyte clone that lysed the original P815 cells and did not lyse normal mouse cells. Clearly, the gene specifying the tumor rejection antigen (named P815A) recognized by these lymphocytes was a logical target for our gene search.

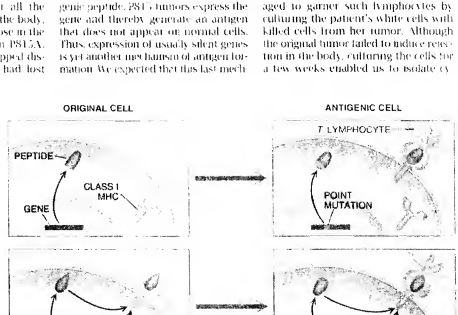
Before starting, however, we wanted to be sure this antigen—which was identified by cytolytic *T* lymphocytes in the test tube—could also direct an immune response to a tumor in the body. We were able to address this question because we had observed an odd effect of P815 cells. Usually when mice are injected with those cells, tumors appear within a month. Yet a few mice formed tumors only after a long delay. When malignancies finally emerged, they resisted attack by the cytolytic *T* lymphocytes responsive to P815A. We concluded correctly, as it turns out, that these animals had rejected almost all the P815 cancer cells because, in the body, *T* lymphocytes identical to those in the clone had recognized antigen P815A. But a few tumor cells had stopped displaying P815A because they had lost the gene specifying it. These so-called antigen-loss variants had proliferated, accounting for the eventual tumor formation. This work demonstrated that an antigen recognized by cytolytic *T* lymphocytes in a laboratory dish might also be of value for eliciting a tumor-rejection response in the body.

Conveniently, such antigen-loss variants could be used as DNA recipients in our efforts to clone the gene coding for antigen P815A. We isolated the gene by applying our by then well-tuned cloning procedure. We built a gene library with DNA from P815 cells and transferred this DNA into cells of an antigen-loss variant. We then picked out the gene from one of the few recipients that incited proliferation of our *T* lymphocytes responsive to antigen P815A. We named the gene *P1A*.

The nucleotide sequence of the *P1A* gene was found to be identical in P815 cells and in normal mouse cells. But in normal cells the gene is inactive; it produces no protein and therefore no antigenic peptide. P815 tumors express the gene and thereby generate an antigen that does not appear on normal cells. Thus, expression of usually silent genes is yet another mechanism of antigen formation. We expected that this last mechanism would generate antigens common to tumors of many different individuals. After all, probably only a relatively limited set of genes can help cancer cells multiply and spread throughout the body. Therefore, we were not surprised to observe that several mastocytoma tumor cell lines express the *P1A* gene, whereas normal mast cells do not.

By 1989 we were ready to begin searching for genes encoding tumor rejection antigens on human cells. We focused on a cell line named *MZ2* MEL, derived from a melanoma tumor (a form of skin cancer) that had formed in a 35-year-old woman known as patient *MZ2*. We isolated a gene on the cell line in much the same way we obtained the mouse *P1A* gene.

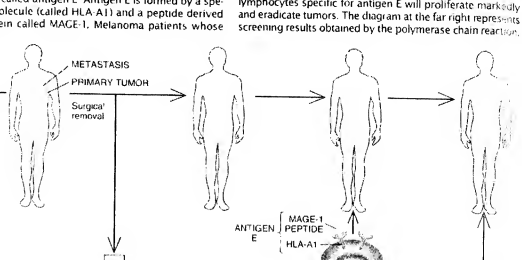
As a first step, we isolated from the patient's white blood cells cytolytic *T* lymphocytes that reacted to the *MZ2*-MEL cells. Like several other groups working with other tumors, we managed to garner such lymphocytes by culturing the patient's white cells with killed cells from her tumor. Although the original tumor failed to induce rejection in the body, culturing the cells for a few weeks enabled us to isolate cy



MUTATION OR ACTIVATION OF CELLULAR GENES can cause cells that do not display antigens recognized by cytolytic *T* lymphocytes (*left column*) to produce antigens (*right column*) that can be recognized by *T* cells.

Scheme for Specific Immunotherapy

One immunotherapy now being considered is based on the discovery that cytolytic *T* lymphocytes isolated from some cancer patients can be induced to react to a molecular complex called antigen E. Antigen E is formed by a specific MHC molecule (called HLA-A1) and a peptide derived from a protein called MAGE-1. Melanoma patients whose cells produce the HLA-A1 molecule (*a*) and whose tumors additionally produce the MAGE-1 protein (*b*) will be injected with killed cells displaying antigen E (*c*). If all goes well (*d*), *T* lymphocytes specific for antigen E will proliferate markedly and eradicate tumors. The diagram at the far right represents screening results obtained by the polymerase chain reaction.



tolytic *T* lymphocytes that selectively lysed the tumor cells. From this potentially mixed population of antitumor lymphocytes, we generated clonal populations that were each reactive to a single antigen.

We also needed an antigen loss variant that could serve as the recipient for DNA from MZ2-MEL cells. This time we obtained the variant by exposing several million MZ2-MEL cells to a similar number of lymphocytes from one cytolytic *T* cell clone—called the anti-E clone because its target antigen was named (arbitrarily) "E." Most of the tumor cells died, but about one in a million lived. These survivors turned out to have lost antigen E. The antigen-loss variants proved sensitive to other *T* cell clones directed against MZ2-MEL cells. Eventually this finding led to the discovery that the MZ2-MEL tumor displays at least four distinct tumor-rejection antigens.

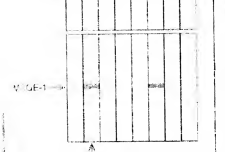
So far we have isolated only the gene that gives rise to antigen E. As might be expected from the *PIA* work, we did so by inserting plasmids carrying the DNA of MZ2-MEL cells into cells of a variant that had lost antigen E. Then we withdrew the gene from one of the few antigen loss cells that activated the anti-E lymphocyte clone. We named the gene *MAGE-1*, for *melanoma antigen-1*.

As soon as we knew the nucleotide sequence of this gene, we rushed to determine whether normal cells of the patient carried the sequence. They did, but the gene was not expressed. Here again a tumor-rejection antigen had arisen through the activation of a gene that is silent in normal cells. This finding illuminated that, in analogy with *PIA*, the gene might be active in tumors of other patients as well. Indeed, analyses of a large selection of tumor samples suggest that more than 30 percent of melanomas carry an active form of the *MAGE-1* gene. More than 15 percent of breast and lung tumors also express the gene. We have not yet discerned how the MAGE-1 protein promotes tumor progression.

Do these figures mean that all patients who express the *MAGE-1* gene also display antigen E on tumor cells? The answer is no, for reasons that have to do with how antigens form. Recall that the *T* cell receptor actually recognizes not a solitary peptide but a complex consisting of a peptide and the surrounding region of the class I MHC molecule. Now, human class I molecules are encoded by three genes (named *HLA-A*, *-B* and *-C*), and these genes are polymorphic, that is, they can differ from one person to another. Each gene, in fact, comes in 10 to 40 different forms, called alleles. Because a person inherits one set of A, B and C alleles from the mother and another set from the father, an individual can manufacture six different varieties of HLA proteins—such as HLA-A1, -A10, -B7, -B24, -C4 and -C6—all of which might differ from the six varieties produced by someone else. The protein products of the alleles differ from one another in the shape of the peptide-binding groove and of the surrounding region. Consequently, in any given cell, a peptide typically binds to only one of the available class I molecules, if it binds at all. Hence, only patients who produce the MAGE-1 protein and a particular HLA molecule will display antigen E. We now know the MHC component of antigen E is HLA-A1. We have also found that the MAGE-1 peptide that binds to this HLA molecule is nine amino acids long, and we know its sequence.

Might patients who lack HLA-A1 but produce the MAGE-1 protein also display antigens that can be recognized by *T* lymphocytes? At this point, we do not know. In theory, such antigens

a test that can detect expression of the genes giving rise to the HLA-A1 and MAGE-1 proteins. Of eight patients tested, three expressed the *HLA-A1* gene and two bore tumors that express the *MAGE-1* gene. Only one individual (patient 2) had positive test results in both categories.



Patient positive for expression of both *HLA-A1* and *MAGE-1* genes

could be created if peptides belonging to the MAGE-1 protein were capable of binding to HLA molecules other than HLA-A1. But we cannot be certain that such antigens exist until we identify cytolytic T lymphocytes that react to them. So far we have been unable to obtain such lymphocytes. The *1* cells that express antigen 1 would not respond to those antigens because they bind only to the specific shape formed by the peptide in antigen 1 and the part of the HLA-A1 molecule that surrounds it.

The identification of the gene coding for a human tumor-rejection antigen opens a new phase in the search for an effective specific immunotherapy for cancer. For the first time, we can select as candidates for therapy those patients who have a chance of benefiting from immunization. We can be selective because it is possible to readily identify individual patients whose tumors carry the known antigen. Further, having the gene for a tumor-rejection antigen means we can devise many innovative ways to immunize patients. Finally, we also have the opportunity to determine rapidly whether the immune system is responding to our interventions, because we can measure changes in the number of a patient's cytolytic T lymphocytes instead of waiting until clinical effects become apparent (such as the absence of relapse).

We are now initiating clinical studies designed to immunize melanoma patients against antigen 1. In these initial studies, we will concentrate on evaluating the cytolytic *T* cell response to the antigen. If we find reliable ways to elicit a good response, later trials will examine cancer remission.

Our methods of identifying candidates for therapy are simpler than might be imagined. We just need to know that their tumors express both the *HLA-A1* and the *MAGE-1* genes. Patients who are about to undergo surgery to remove a tumor can be tested for their HLA type in a couple of ways. One of these methods, based on a small sample of blood, yields results in a few hours. In individuals who test positive for HLA-A1, a sample of tumor can be frozen immediately after surgery. Within two days, a sophisticated technique called the polymerase chain reaction will reveal whether the tumors also express the *MAGE-1* gene [see "The Unusual Origin of the Polymerase Chain Reaction," by Kary B. Mullis; SCIENTIFIC AMERICAN, April 1990]. About 26 percent of white individuals and 17 percent of black individuals carry the *HLA-A1* allele. Considering that some 46 percent of melanoma patients express the *MAGE-1* gene, we can predict that roughly 8 percent of melanoma patients will display antigen E on their tumor cells.

A number of immunization modes can be tested on candidates who fit our dual criteria. Because the *MAGE-1* gene and the antigenic peptide have been identified, we can induce various cell types to express antigen E. Killed versions of the cells can be injected into patients to spur their anti-E lymphocytes into action. Our first clinical studies will follow such a protocol.

We also hope to evaluate the effectiveness of inserting a gene for an interleukin, such as interleukin-2, into cells expressing antigen E. The interleukin should facilitate the activation of *T* lymphocytes around these cells. Synthetic E peptides or purified MAGE-1 proteins that have been mixed with an immune stimulatory substance called an adjuvant will also be tried. Finally, we might insert the *MAGE-1* gene into the DNA of a harmless virus that can penetrate into human cells but cannot reproduce there. After such recombinant viruses are administered to patients, a relatively small number of cells should become infected. These cells should produce the MAGE-1 protein and display antigen E for a while. Immunization with peptides, proteins and recombinant viruses has already proved quite effective for other purposes.

I do not know whether these treatments will cure patients, but I believe there is a good chance that some form of specific immunotherapy will be helpful. My associates and I are encouraged by mouse studies in which strong anti-tumor responses have been obtained without hurting the general health of the animals. But it is difficult to predict whether the specific immunotherapies I have described will eradicate human cancers, particularly in patients who harbor large tumors. Malignant cells that have lost the ability to produce the MAGE-1 or HLA-A1 protein may arise. Such cells would no longer make antigen E and would thus escape notice of the anti-E lymphocytes. Success, then, may have to wait until we can immunize cancer patients with several tumor-rejection antigens simultaneously. These multiple immunizations should strengthen the immune reaction and also help to prevent variants that have lost one antigen from escaping destruction.

We are confident that the gene-cloning techniques we have developed will lead in the near future to identification of additional genes specifying tumor rejection antigens. The advances will make it feasible to attack tumors through several antigens. And they will render increasing numbers of patients eligible for trials of specific immunotherapies. Thus, even though success is by no means assured and the work ahead remains considerable, a clear strategy has now been mapped out for the specific immunotherapy of cancer.

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